Standard Test Method for Determining the Aerobic, Aquatic Biodegradability of Lubricants or Lubricant Components in a Closed Respirometer[1]

This standard is issued under the fixed designation D6731; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers a procedure for determining the degree of biodegradability of lubricants or their components in an aerobic aqueous medium on exposure to an inoculum under controlled laboratory conditions. This test method is an ultimate biodegradation test that measures oxygen demand in a closed respirometer.

1.2 This test method is suitable for evaluating the biodegradation of volatile as well as nonvolatile lubricants or lubricant components.

1.3 This test method is applicable to lubricants and lubricant components which are not toxic and not inhibitory to the test microorganisms at the test concentration.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazards are given in Section 10.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D1293 Test Methods for pH of Water

D4175 Terminology Relating to Petroleum, Petroleum Products, and Lubricants

D4447 Guide for Disposal of Laboratory Chemicals and Samples

D6384 Terminology Relating to Biodegradability and Ecotoxicity of Lubricants

E943 Terminology Relating to Biological Effects and Environmental Fate

2.2 *ISO Standards:*[3]

ISO 4259:1992(E) Petroleum Products–Determination and Application of Precision Data in Relation to Methods of Test

ISO 6107-2:1997 Water Quality–Vocabulary–Part 2

ISO 8192:1986 Water Quality–Test for Inhibition of Oxygen Consumption by Activated Sludge

ISO 9408:1999 Water Quality–Evaluation of Ultimate Aerobic Biodegradability of Organic Compounds in Aqueous Medium by Determination of Oxygen Demand in a Closed Respirometer

2.3 *OECD Standards:*[4]

OECD 301F:1992 Ready Biodegradability-Manometric Respirometry

2.4 *APHA Standards:*[5]

2540B Total Solids Dried at 103-105°C

9215 Heterotrophic Plate Count

3. Terminology

3.1 *Definitions:*

3.1.1 Definitions of terms applicable to this test method appear in the Compilation of ASTM Standard Definitions and the following terminology standards: D1129, D4175, D6384, E943, and ISO 6107-2:1997.

3.1.2 *activated sludge, n*—the precipitated solid matter, consisting mainly of bacteria and other aquatic microorganisms, that is produced at a domestic wastewater treatment plant and is used primarily in secondary sewage treatment to microbially oxidize dissolved organic matter in the effluent.

[1] This test method is under the jurisdiction of ASTM Committee D02 on Petroleum Products and Lubricants and is the direct responsibility of Subcommittee D02.12 on Environmental Standards for Lubricants.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute, 25 W. 43rd St., 4th Floor, New York, NY 10036.

[4] Available from the Head of Publications Service, Organization for Economic Cooperation and Development (OECD), 2, rue Andre-Pascal, 75775 Paris Cedex 16, France.

[5] From Standard Methods for the Examination of Water and Wastewater, latest edition. Available from the American Public Health Assoc., 1015 18th St., NW, Washington, DC 20036.

3.1.3 *aerobic*, *adj*—(*a*) taking place in the presence of oxygen; (*b*) living or active in the presence of oxygen.

3.1.4 *biochemical oxygen demand (BOD)*, *n*—the mass concentration of dissolved oxygen consumed under specified conditions by the biological oxidation of organic or inorganic matter, or both.

3.1.4.1 *Discussion*—BOD determination is performed using empirical tests employing standardized laboratory procedures. These tests measure oxygen utilization during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) in water.

3.1.5 *biodegradation*, *n*—the process of chemical breakdown or transformation of a test material caused by microorganisms or their enzymes.

3.1.5.1 *Discussion*—Biodegradation is only one mechanism by which materials are removed, transformed, or both, in the environment.

3.1.6 *lag phase*, *n*—the period of diminished physiological activity and cell division following the addition of microorganisms to a new culture medium.

3.1.7 *log phase*, *n*—the period of growth of microorganisms during which cells divide at a positive constant rate.

3.1.8 *pre-adaptation*, *n*—the incubation of an inoculum in the presence of the test material which is done prior to the initiation of the test and under conditions similar to the test conditions.

3.1.8.1 *Discussion*—The aim of pre-adaptation is to improve the precision of the test method by decreasing variability in the rate of biodegradation produced by the inoculum. Pre-adaptation may mimic the natural processes which cause changes in the microbial population of the inoculum leading to more rapid biodegradation of the test material but is not expected to change the overall extent of biodegradation of the test material.

3.1.9 *pre-condition*, *n*—the pre-incubation of an inoculum under the conditions of the test in the absence of the test material.

3.1.10 *sludge*, *n*—a water-formed sedimentary deposit.

3.1.11 *suspended solids (of an activated sludge or other inoculum samples)*, *n*—solids present in activated sludge or other inoculum samples that are not removed by settling under specified conditions.

4. Summary of Test Method

4.1 Biodegradation of a lubricant or the component(s) of a lubricant is determined by measuring the oxygen consumed when the lubricant or component is exposed to microorganisms under controlled aerobic aquatic conditions. This value is then compared to the theoretical amount of oxygen (ThO_2) which is required to oxidize all of the elements (that is, carbon, hydrogen, nitrogen, and so forth) in the test material. This test method mixes the test material (lubricant or component) with aerobic microorganisms in a closed respirometer containing a defined aquatic medium and measures the biodegradation of the test material by following the decrease in oxygen in the respirometer.

4.2 The test material is the sole source of carbon and energy in the medium. A reference material known to biodegrade, such as low erucic acid rapeseed oil (LEAR or canola oil) is run alongside the test material to confirm that the inoculum is viable and capable of biodegrading suitable materials under the test conditions. The test material or reference material concentration is normally 50 to 100 mg/L, providing a theoretical oxygen demand of at least 50 mg O_2/L but no more than 200 mg O_2/L. The ThO_2 of the test and reference materials will be determined from measured elemental compositional analysis and will be calculated as in 13.1.

4.3 The inoculated medium is stirred in a closed flask and the consumption of oxygen is determined either by measuring the amount of oxygen required to maintain a constant gas volume in the respirometer flask, or by measuring the change in volume or pressure (or a combination of the two) in the apparatus.

4.4 Evolved CO_2 (carbon dioxide) is absorbed in an alkaline trap solution (for example, 10 *M* NaOH or KOH) or other CO_2-absorbing system suspended within the test vessel, typically in the headspace of the test vessel.

4.5 Biodegradation is followed over a specified period by determining the consumption of oxygen. The amount of oxygen utilized in oxidation of the test and reference material is corrected for oxygen uptake by the inoculum in the blank controls and is expressed as a percentage of the theoretical oxygen demand (ThO_2) calculated from the empirical formula of the material. Evaluation of the biodegradability of the test material is made on the basis of these data. Normally the test duration is 28 days; however, the test may be terminated if oxygen consumption has plateaued. The test may be extended as long as the systems' integrity is maintained and the inoculum in the blank systems is viable. The duration of the test will be dependent on the length of time required for the rate of test material biodegradation to achieve a plateau. A graphical illustration of the test results for a biodegradable material is presented in Fig. 1.

5. Significance and Use

5.1 Results from this test method suggest the degree of aerobic, aquatic biodegradation of a lubricant or lubricant component. The rate and extent of oxygen consumption is measured upon exposure of the test material to an inoculum within the confines of a controlled laboratory setting. Test materials which achieve a high degree of biodegradation in this test may be assumed to easily biodegrade in many aerobic aquatic environments.

5.2 Because of the stringency of this test method, low results do not necessarily mean that the test material is not biodegradable under environmental conditions, but indicate that further testing is necessary to establish biodegradability.

5.3 If the pH value at the end of the test is outside the range from 6 to 8 and if the percentage degradation of the test material is less than 50 %, it is advisable to repeat the test with a lower concentration of the test material or a higher concentration of the buffer solution, or both.

5.4 A reference or control material known to biodegrade under the conditions of this test method is necessary in order to verify the activity of the inoculum. The test must be regarded as invalid and shall be repeated using a fresh inoculum if the reference material does not demonstrate biodegradation to the extent of >60 % of the ThO_2 within 28 days.

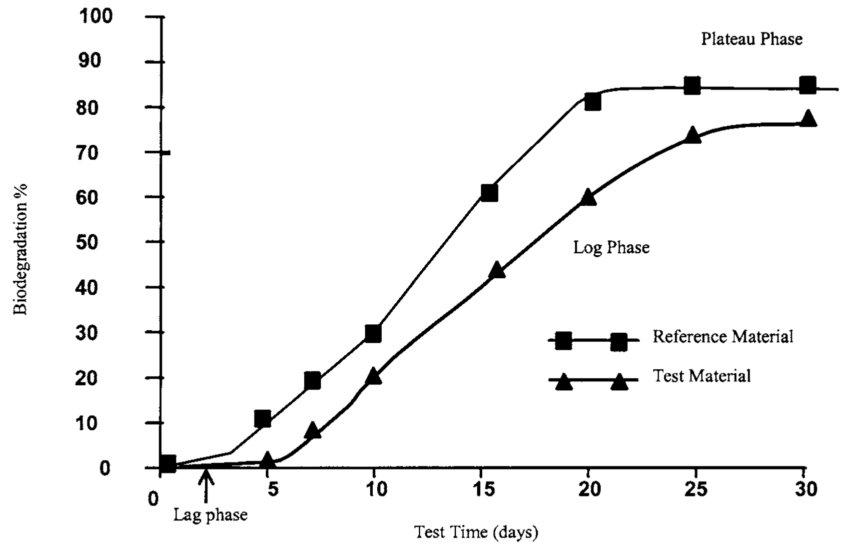

FIG. 1 Respirometric Test–Biodegradation Curve

5.5 Information on the toxicity of the test material to the inoculum may be useful in the interpretation of low biodegradation results. Toxicity of the test material to the inoculum may be evaluated by testing the test material in combination with the reference material in inhibition control systems. If an inhibition control is included, the test material is assumed to be inhibiting if the degradation percentage of the reference material is lower than 40 % (ISO 8192:1986). In this case, it is advisable to repeat the test with lower concentrations of the test material.

5.6 Total oxygen utilization in the blank at the end of the test exceeding 60 mg O_2/L invalidates the test.

5.7 The water solubility or dispersibility of the lubricant or component may influence the results obtained and hence comparison of test results may be limited to lubricants or components with similar solubilities.

5.8 The behaviors of complex mixtures are not always consistent with the individual properties of the components. Test results for individual lubricant components may be suggestive of whether a mixture containing these components (that is, fully formulated lubricants) is biodegradable, but such information should be used judiciously.

6. Apparatus

6.1 *Closed Respirometer*:

6.1.1 The principle of a closed respirometer is given in Fig. 2. When testing volatile compounds, the apparatus used shall be appropriate or adapted to this particular purpose in accordance with the manufacturer's instructions. Exercise care that the closed respirometer apparatus is well sealed to prevent any loss (for example, leakage) of volatile compounds from the system or of oxygen into the system.

6.1.2 The test mixture is stirred by a magnetic stirrer in the test flask, which is filled with sufficient volume to minimize headspace and prevent delay of O_2 and CO_2 diffusion through the air-water phases. This volume is dependent on the selected flask size, and is normally specified by the manufacturer of the respirometer. If biodegradation takes place, the microorganisms consume oxygen and produce carbon dioxide. Oxygen from the headspace is then dissolved in the liquid to reestablish chemical equilibrium. The carbon dioxide produced by the microorganisms diffuses into the headspace where it is trapped in an absorbent solution or material and the total pressure in the flask then decreases.

6.1.3 This pressure drop is detected by a manometer, which produces a signal that results in the electrolytic generation of oxygen. When the original pressure is re-established, the signal is stopped and the quantity of electricity used is measured. The amount of electricity used is proportional to the amount of consumed oxygen. This is indicated on a plotter or a printer, or the data are collected using an appropriate software program.

6.2 *Water-Bath or Constant Temperature Room*, to comply with 11.2.

6.3 *Centrifuge*.

6.4 *pH-meter*.

6.5 *Analytical Balance*, capable of weighing to appropriate precision and accuracy (for example, ±0.0001 g).

7. Reagents and Materials

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[6] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without decreasing the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification D1193.

[6] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

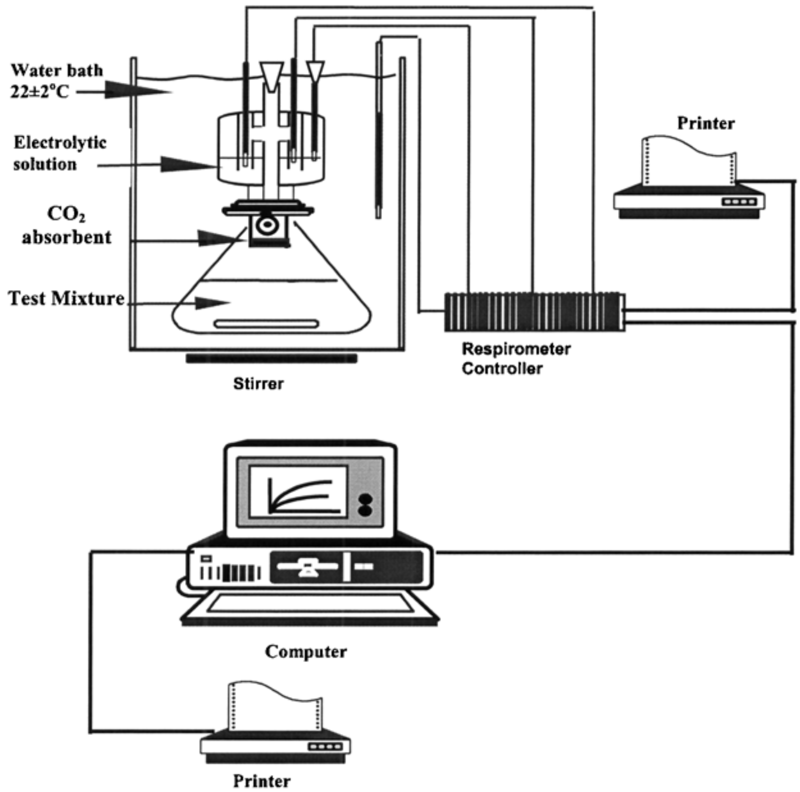

FIG. 2 Principle of a Closed Respirometer

7.3 Prepare the following stock solutions:

7.3.1 *Calcium Chloride Solution*—Dissolve 27.5 g of anhydrous calcium chloride ($CaCl_2$) or 36.4 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in water and dilute to 1 L.

7.3.2 *Ferric Chloride Solution*—Dissolve 0.25 g of iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in water and dilute to 1 L. Prepare this solution just before use or add a drop of concentrated hydrochloric acid (HCl) or 0.4 g/L of ethylenediamine-tetraacetic acid (EDTA).

7.3.3 *Magnesium Sulfate Solution*—Dissolve 22.5 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in water and dilute to 1 L.

7.3.4 *Phosphate Buffer Solution*—Dissolve 8.5 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4), 21.75 g anhydrous potassium monohydrogen phosphate (K_2HPO_4), 33.4 g disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), and 0.5 g ammonium chloride (NH_4Cl) in water and dilute to 1 L. Alternatively, 50.3 g of disodium hydrogen phosphate, heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) may be used in place of $Na_2HPO_4 \cdot 2H_2O$. The pH of this solution shall be about 7.4.

8. Inoculum Test Organisms

8.1 *Sources of the Inoculum*—Activated sewage-sludge from a sewage-treatment plant that treats principally domestic waste may be considered as an aerobic inoculum. An inoculum derived from soil or natural surface waters, or any combination of the three sources, may also be used in this test method. Allowance for various and multiple inoculum sources provides access to a greater diversity of biochemical competency and potentially represents more accurately the capacity for biodegradation. The following provides several options for where and how to obtain an appropriate inoculum:

8.1.1 *Inoculum from Activated Sludge*—Activated sludge freshly sampled (that is, less than 24 h old) from a well-operated predominantly domestic sewage treatment plant (that is, one with no recent upsets and operating within its design parameters) may be used. This sewage treatment plant should receive minimal or no effluent from industry.

8.1.1.1 Using CO_2-free air, aerate sludge in the laboratory for 4 h. Depending on the number of test systems, sufficient volume of the mixed liquor is sampled and homogenized for 2 min at medium speed using a high-sheer/high-speed blender. Withdraw a sample for the determination of the dry weight of the suspended solids (8.2.2). Keep the inoculum continuously well mixed until all sample preparation is completed to avoid solids settling.

8.1.1.2 Calculate the volume of homogenized mixed liquor necessary to achieve a final sludge dry-weight concentration in the test medium of 30 mg/L (suspended solids, 8.2.2). The inoculum prepared from the homogenized mixed liquor may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.1), or added directly to the test systems (12.2).

8.1.1.3 Alternatively, settle the homogenized sludge for 30 min or longer (if required) and decant the liquid supernatant for use as inoculum. The inoculum prepared from the supernatant may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.2), or added directly to the test systems (12.2).

8.1.1.4 It is optional to pre-condition the inoculum. Preconditioning consists of aerating the activated sludge for up to seven days. Sometimes pre-conditioning improves the precision of the test method by reducing the amount of oxygen consumption in the blank controls.

NOTE 1—Exercise care in pre-conditioning because of the sensitivity of inocula to prolonged aeration and starvation conditions. Pre-conditioning should be applied in situations where it is known that the inoculum source consistently shows a high internal respiration rate.

8.1.2 *Inoculum from Secondary Effluent*—Alternatively, the inoculum can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving domestic sewage.

8.1.2.1 Allow the secondary effluent to settle for 1 h and collect the supernatant or filter the effluent through a coarse filter paper. After supernatant collection or effluent filtration, aerate the sample using CO_2-free air in the laboratory for 4 h. The inoculum may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.2), or added at this point to the test systems (12.2). Up to 100 mL of this type of inoculum may be used per litre of medium.

8.1.3 *Inoculum from Surface Water*—A further source for the inoculum is surface water. In this case, collect a sample of an appropriate surface water (for example, river or lake) and keep aerobic until required.

8.1.3.1 Filter the surface water through a coarse filter paper or glass wool plug, and discard the first 200 mL. Aerate the remaining filtered sample using CO_2-free air in the laboratory for 4 h. The inoculum may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.1), or added directly to the test systems (12.2). Up to 100 mL of this type of inoculum may be used per litre of medium.

8.1.4 *Inoculum from Soil*:

8.1.4.1 Suspend 100 g of soil in 1000 mL of water.

8.1.4.2 Allow the suspension to settle for 30 min.

8.1.4.3 Filter the supernatant through a coarse filter paper or glass wool plug, and discard the first 200 mL. The filtrate is aerated immediately and continuously until used. The soil inoculum may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.1), or added directly to the test systems (12.2). Up to 100 mL of this type of inoculum may be used per litre of medium.

8.1.5 *Composite Inoculum*—The four inoculum sources may be combined in any proportion and mixed well.

8.2 *Determination of Microorganisms*:

8.2.1 APHA Test Method 9215, or equivalent, shall be used to enumerate the microorganisms in the inoculum. The inoculum shall contain 10^6 to 10^7 colony-forming units per millilitre. It is optional to measure the total bacterial count of the inoculum using the dip slide technique with a commercially available diagnostic kit.

8.2.2 Alternatively for inoculum from activated sludge, APHA Test Method 2540B shall be used to determine the sludge dry-weight per unit volume. Calculate the volume of mixed liquor necessary to achieve a final sludge dry-weight concentration in the test medium of 30 mg/L (suspended solids).

8.3 Pre-adaptation of any inoculum type to a test material is allowed. A sufficient volume of pre-adapted inoculum in test medium shall be incubated for 14 days to yield a minimum of 100 mL of inoculated medium for each respirometer test system; that is, 100 mL for each blank, test material, and positive control material replicate. When developing pre-adapted inoculum for more than one test material, individual cultures will be prepared separately for each test material. Pre-adaptation can be accomplished as follows:[7]

8.3.1 *Pre-adaptation of Homogenized Mixed Liquor Inoculum*—Supplement the calculated volume of homogenized mixed liquor inoculum necessary to achieve a suspended solids concentration of 30 mg/L (8.1.1.2) with 25 mg/L of vitamin-free casamino acids and 25 mg/L of yeast extract.

8.3.1.1 Add the supplemented inoculum to a 2-L Erlenmeyer flask. Add 10 mL of phosphate buffer solution, 1 mL of magnesium sulfate solution, 1mL of ferric chloride solution, and 1mL of calcium chloride solution to the 2-L Erlenmeyer flask. Add sufficient volume of water to the 2-L Erlenmeyer flask to achieve a total volume of 1000 mL. Prepare separate inoculated test medium for each test material requiring pre-adaptation.

8.3.2 Pre-adaptation of inoculum prepared from one of the following sources: activated sludge supernatant, 8.1.1.3; secondary effluent, 8.1.2.1; surface water, 8.1.3.1; soil, 8.1.4.3; or composite, 8.1.5. Supplement inoculum with 25 mg/L of vitamin-free casamino acids and 25 mg/L of yeast extract.

8.3.2.1 Add 100 mL of the supplemented inoculum prepared in 8.3.2 to a 2-L Erlenmeyer flask. Add 10 mL of phosphate buffer solution, 1mL of magnesium sulfate solution, 1mL of ferric chloride solution, and 1mL of calcium chloride solution to the 2-L Erlenmeyer flask. Add sufficient volume of water to the 2-L Erlenmeyer flask to achieve a total volume of 1000 mL. Prepare separate inoculated test medium for each test material requiring pre-adaptation.

8.3.3 The inoculum flasks are maintained at a temperature of 22°C (±2°C) in the dark and are agitated on a shaker or shaker table or with magnetic stirrers at a moderate speed (for example, 150 to 200 rpm). Add test materials incrementally during the acclimation period at concentrations equivalent to 4, 8, and 8 mg carbon/L on Days 0, 7 and 11, respectively, to ensure the use of a consistent amount of test material.

8.3.4 On Day 14, homogenize the culture in a blender for at least 1 min and refilter the medium through glass wool prior to use as the inoculum for the test. If pre-adaptation is conducted for a series of functionally or structurally related materials, media from the separately prepared flasks may be combined before final filtration.

9. Test Material and Reference Material

9.1 This section addresses specific requirements pertaining to the theoretical oxygen demand (ThO_2) of the test material and reference material as well as the appropriate choice of the reference material.

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[7] Sturm, R. N., "Biodegradability of Nonionic Surfactants: Screening Test for Predicting Rate and Ultimate Biodegradation," *Journal of American Oil Chemists Society*, Vol 50, 1973, pp. 159-167.

9.2 The theoretical oxygen demand (ThO_2) shall be determined based on results of elemental analysis and calculations in 13.1.

9.3 The test material shall be added to the appropriate respirometer test systems to obtain a loading of 50 to 100 mg/L and a test material ThO_2 requirement of 50 to 200 mg O_2/L in the test medium.

9.4 *Reference*—A material known to be biodegradable shall be tested simultaneously with the test material.

9.4.1 For water soluble test materials, suggested reference materials are sodium benzoate and aniline.

9.4.2 For water insoluble test materials, the suggested reference material is low erucic acid rapeseed oil, also called LEAR or canola oil. The low erucic acid rapeseed oil shall contain a maximum of 2 % by weight erucic acid.

9.4.3 The reference material will be added to the appropriate respirometer test systems to obtain a loading of 50 to 100 mg/L, in order to require 50 to 200 mg O_2/L as reference material ThO_2 in the test medium in the same manner as the test material.

9.4.4 The results from flasks containing the reference material verify the viability of the inoculum.

9.5 The test method shall be performed in a minimum of two replicate test systems on all test and reference materials, but triplicates are preferred.

10. Hazards

10.1 This test method includes the use of hazardous chemicals. Avoid contact with chemicals and follow the manufacturer's instructions and Material Safety Data Sheets.

10.2 This test method includes the use of potentially harmful microorganisms. As such, execution of this test method must be carried out under the guidance of qualified personnel who understand the safety and health aspects of working with microorganisms. Minimally, review this test method with an industrial hygienist before initiating any activity. Avoid contact with the microorganisms by using gloves and other appropriate protective equipment and sterile procedures. Use good personal hygiene to minimize exposure to harmful microbial agents.

10.3 Whenever appropriate, materials and supplies contaminated with biologically active cultures should be sterilized or autoclaved before discarding or reusing them.

10.4 Chemicals should be disposed of as described in Guide D4447 or as prescribed by current regulations.

11. Preparation of Apparatus

11.1 *Cleaning*—The following is a suggested method for cleaning glassware and equipment to avoid organic contamination which may affect test results. The flasks and equipment used to prepare and store stock solutions and test solutions should be cleaned before use. Items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid), and at least twice with distilled deionized water. Some organic solvents might leave a film that is insoluble in water. The presence of this film is not acceptable and may lead to false positive results. At the end of every test, all items that are to be used again should be immediately (*a*) emptied, (*b*) rinsed with water, and (*c*) cleaned as previously stated.

11.2 *Test Environment*—Incubation shall take place in the dark or in diffused light, in an enclosure that is maintained at a constant temperature (within ±1°C) between 20 and 24°C and which is free from toxic vapors.

12. Procedure

12.1 Set up the closed respirometer (see the examples described in 6.1, manufacturer's manual, ISO 9408:1999, or OECD 301F:1992). For each blank, test material, and reference material being tested, prepare the following (based on 1–L test system volume):

12.1.1 Prepare the test medium by adding 850 mL of water to each of the respirometer flasks. To each of the respirometer flasks, add 10 mL of the phosphate buffer solution, 1mL of magnesium sulfate solution, 1 mL of ferric chloride solution, and 1 mL of calcium chloride solution.

12.2 *Addition of Inoculum*:

12.2.1 *Addition of Non-adapted Homogenized Mixed Liquor*—Add sufficient volume of the homogenized mixed liquor inoculum (8.1.1.2) to each of the respirometer flasks to give 30 mg/L of suspended solids.

12.2.2 Addition of non-adapted activated sludge supernatant (8.1.1.3); secondary effluent (8.1.2.1); surface water (8.1.3.1); soil (8.1.4.3); or composite (8.1.5). Add 10 mL of the selected inoculum to each of the respirometer flasks.

12.2.3 *Addition of Adapted Inoculum*—Add 100 mL of pre-adapted inoculum (8.3.4) to each of the respirometer flasks (see Test Method D1293).

12.3 Measure the pH in each flask and adjust to pH 7.4 ± 0.2, if necessary, with dilute HCl or NaOH before adding the test material or reference material.

12.4 *Addition of Test Material or Reference Material*:

12.4.1 The concentration of the test material or reference material in the test medium shall be approximately 50 to 100 mg/L, providing at least 50 mg ThO_2 in the test medium, but no more than 200 mg ThO_2. Calculate the ThO_2 to ensure this is within the range specified. Decrease or increase the amount of material necessary to achieve a ThO_2 within the specified range.

12.4.2 Add the test material or reference material gravimetrically to the replicate respirometer flasks. If, in order to accomplish this, the material is weighed into or onto a small object, then both the material and object shall be added to the flask.

NOTE 2—An example of a small object might be a glass fiber filter. The test or reference material is added to the respective shake flasks as a measured weight adsorbed to the surface of the filter. This enables an accurate weight to be dosed into each flask and increases the surface area of the material. A blank glass fiber filter should also be added to each blank flask.

12.4.3 Sonication of the test material or reference material in 5 mL of water while still in or on a small object is allowed as a means of obtaining a better dispersion of insoluble materials in the test medium. If sonication is performed, the object shall also be added to the flask. In addition, if sonication is performed on the test material, the reference material shall also be sonicated in an identical manner prior to its addition to the test medium.

12.4.4 Along with the flasks containing test material or reference material, additional replicate flasks shall contain the test medium and the inoculum with no other additions. These flasks shall be blanks.

12.4.5 Add sufficient volume of water to achieve a final volume of 1000 mL in each flask.

12.4.6 Add sufficient alkaline solution (10 *M* NaOH or KOH), or other suitable absorbent to the CO_2-absorber compartments of the respirometer. These solutions may be prepared in the laboratory or obtained commercially. A 10 *M* NaOH solution is prepared by cautiously dissolving 400 g NaOH in distilled water to a final volume of 1 L. A 10 *M* KOH is prepared by dissolving 658.8 g KOH in distilled water to a final volume of 1 L. Filter each solution to free it of solid material, confirm molarity by titration with standard acid, and store under nitrogen, sealed to prevent absorption of CO_2 from the air.

12.4.7 Seal the flasks and, in the case of automatic respirometers, perform any procedures as specified in the manufacturer's manual to initiate oxygen consumption measurements, and start stirring the contents of each flask.

12.4.8 Incubation shall take place in the dark or diffused light, in an enclosure that is maintained at a constant temperature (within at least 1°C) between 20 and 24°C. Record the test temperature throughout the test.

12.4.9 Take necessary readings on the manometers (if manual) and verify that the oxygen consumption data is being recorded properly (automatic respirometer).

12.4.10 Stop the test after the specified period, (usually 28 days), or earlier if a plateau of oxygen consumption has been attained. The test may be extended as long as the systems' integrity is maintained and the inoculum in the blank system is viable. The duration of the test will be dependent on the length of time required for the rate of test material biodegradation to achieve a plateau.

12.4.11 Measure the final pH value of the flask contents at the end of the test.

13. Calculation and Expression of Results

13.1 *Calculation*:

13.1.1 The ThO_2 of the material $C_c\ H_h\ Cl_{cl}\ N_n\ Na_{na}\ O_o\ P_p\ S_s$, of empirical weight M_r, can be calculated according to:

$$ThO_2 = \frac{16[2c+1/2(h-cl-3n)+3s+5/2p+1/2na-o]}{M_r} \quad (1)$$

13.1.2 This calculation implies that C is mineralized to CO_2, H to H_2O, P to P_2O_5, and Na to Na_2O. The Cl is eliminated as hydrogen chloride and nitrogen as ammonia. Sulfur is assumed to be oxidized to the S^{+6} oxidation state.

13.1.2.1 Example of the calculation of the theoretical oxygen demand: glucose ($C_6H_{12}O_6$), M_r = 180 g/mol.

$$ThO_2 = \frac{16[2*6+(1/2*12)-6]}{180} = 1.07\ mg\ O_2/mg\ glucose \quad (2)$$

13.1.2.2 Empirical molecular weights of salts other than those of the alkali metals are calculated on the assumption that these salts have been hydrolyzed.

13.1.3 Example of the calculation of the theoretical oxygen demand: sodium *n*-dodecylbenzenesulfonate ($C_{18}H_{29}SO_3Na$), M_r = 348 g/mol

$$ThO_2 = \frac{16(36+29/2+3+1/2-3)}{348} = 2.34\ mg\ O_2/mg\ compound \quad (3)$$

13.2 Calculate the oxygen consumption values for each flask at selected time intervals, from the reading obtained, using the method given by the manufacturer for the appropriate type of respirometer. Calculate the oxygen demand in milligrams per litre of the test material as the difference between oxygen consumption in the test flask and the mean oxygen consumption of the blank flasks. Divide this difference by the concentration of the test material to give the net oxygen consumption expressed as specific BOD in milligrams of O_2 per milligram of test material.

13.2.1 Specific BOD at selected time intervals = $OD_t - OD_{Bl,\,t} / \rho TC$

where:

OD_t = oxygen consumption of the test material solution at time, *t*, mg/L,
$OD_{Bl,\,t}$ = mean oxygen consumption of the blanks at time, *t*, mg/L, and
ρTC = mass concentration of the test material, mg/L.

13.2.2 The degradation is defined as the ratio of the specific biochemical oxygen demand to the theoretical oxygen demand (ThO_2). Determine the percentage degradation (D_t) for each test flask, using the following equation:

$$D(ThO_2)_t = \frac{BOD}{ThO_2} \times 100 \quad (4)$$

where:

$D(ThO_2)_t$ = percentage biodegradation of ThO_2 at time *t*.

13.2.3 These calculations may be automatically generated by specific commercial respirometers or by specific software data analysis systems interfaced to the respirometer.

13.3 Plot the percentage degradation, D_t for each flask against time to obtain the degradation curve (see example in 4.5). Draw an average curve if comparable results in the parallel test flasks are obtained.

13.4 If sufficient data are available, indicate clearly on the curve the lag time, the maximum level of degradation, and the degradation time.

14. Report

14.1 Report the following data and information:

14.1.1 Information on the inoculum, including source, date of collection, storage, handling, and if used, the method of pre-adaptation to the test material.

14.1.2 Method and results of determination of microorganisms.

14.1.3 Identification of the reference material.

14.1.4 Elemental analysis (giving percentage composition of all elements present), or empirical formula used to derive ThO_2 of the test material and reference material.

14.1.5 Information on preparation of the test material and reference material, including any procedures for enhancing their dispersion into the test medium.

14.1.6 Main characteristics of the respirometer (manufacturer's name and address where appropriate).

14.1.7 Percentage of ThO_2 achieved at the plateau and the number of days to reach the plateau.

14.1.8 Percentage of ThO_2 achieved for each test material and reference material by the end of the test and the length of the test.

14.1.9 The cumulative average of percent ThO_2 over time should be displayed graphically for each test and reference material because the lag-phase, that is, the delay in the onset of biodegradation as well as the rate of biodegradation are important. It is optional to graphically plot the percentage of ThO_2 achieved over time for the individual replicates.

14.1.10 The replicate standard deviation (if applicable) for each test and reference material. The average of all replicates unless one or more replicates may be excluded based on statistical grounds as given in ISO 4259:1992(E). In that case, report the excluded data and the reason for exclusion.

14.1.11 The temperature range of the test.

14.1.12 Initial and final pH.

15. Precision and Bias

15.1 The precision and bias of the procedure in this test method for measuring the aerobic aquatic biodegradability of lubricants or their components is not determined as of yet.

16. Keywords

16.1 aerobic; biodegradation; BOD; degree (of biodegradation); municipal; respirometer; sewage sludge; ThO_2; water insoluble